

Delayed Treatment with Lithospermate B Attenuates Experimental Diabetic Renal Injury

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Abstract. Extracellular matrix (ECM) accumulation in the glomerular mesangium is a characteristic feature of diabetic nephropathy. While transforming growth factor- β 1 (TGF- β 1) is the final mediator of ECM accumulation, reactive oxygen species (ROS) and protein kinase C (PKC) are the upstream signaling molecules that mediate hyperglycemia-induced ECM expansion. Magnesium lithospermate B (LAB) is an active component isolated from *Salvia miltiorrhizae* with known renoprotective properties due to its antioxidative effects. Thus, the present study examined the effects of LAB on renal injury in streptozotocin-induced diabetic rats (STZR) and on the activation of mesangial cells cultured under high glucose conditions. Ten micrograms of LAB/kg per day was started 8 wk after streptozotocin injection and continued for a period of 8 wk. It significantly suppressed renal malondialdehyde (MDA), microalbuminuria, glomerular hypertrophy, mesangial expansion,

and the upregulation of renal TGF- β 1, fibronectin, and collagen in STZR without significantly affecting plasma glucose. Both 30 mM of glucose and 100 μ M of H₂O₂ significantly increased TGF- β 1 and fibronectin protein secretion by mesangial cells. LAB at 10 μ g/ml inhibited high glucose- and H₂O₂-induced TGF- β 1 and fibronectin secretion. LAB also inhibited glucose-induced intracellular ROS generation and PKC activation in mesangial cells, but it did not directly inhibit PKC activity at dosages that inhibited ROS generation. The *in vitro* data of this study show that LAB inhibits ROS generation leading to PKC activation and TGF- β 1 and fibronectin upregulation in mesangial cells cultured under high glucose conditions. Moreover, delayed treatment with LAB was found to significantly suppress the progression of renal injury in STZR. LAB may become a new therapeutic agent for the treatment of diabetic nephropathy.

Diabetic nephropathy is characterized functionally by glomerular hyperfiltration and albuminuria and histologically by the expansion of the glomerular mesangium, which is related to the loss of renal function (1).

High glucose (30 mM) is the main determinant of diabetic nephropathy in both type 1 (2) and type 2 (3) diabetes. Though transforming factor- β 1 (TGF- β 1) is the final mediator of extracellular matrix (ECM) expansion in diabetic nephropathy (4–6), increased reactive oxygen species (ROS) generation (7,8) and protein kinase C (PKC) activation (9,10) in association with increased glucose metabolism are considered to be the main upstream signaling molecules of glucose-induced vascular injury, which includes diabetic nephropathy.

ROS generation (8,11,12) and PKC activation (13–15) are elevated in mesangial cells cultured under high glucose con-

ditions. The kidneys of experimental diabetic animals also exhibit increased lipid peroxidation (16), which is a marker of increased ROS generation, and PKC activation (16). In addition, exogenously administered ROS (8,17) and PKC activator (8) lead to TGF- β 1 and ECM upregulation in mesangial cells. Moreover, antioxidants (18–26) and PKC inhibition (27,28) have been reported to prevent or attenuate both glucose-induced mesangial cell activation and renal injury in diabetes.

Salvia miltiorrhizae radix is a Chinese herbal medicine that has been traditionally used for the treatment of diabetic complications. Lithospermate B (LAB) was recently isolated from *S. miltiorrhizae* and found to have beneficial effects on the renal function of nephrectomized rats (29), possibly by reducing ROS generation (30). It is tetramer of caffeic acid (Figure 1). Thus, the present study was undertaken to examine the effects of delayed LAB treatment on the renal function of streptozotocin-induced diabetic rats (STZR) and to evaluate the role of the antioxidative effects of LAB on protection against diabetic renal injury using mesangial cells cultured under high glucose conditions.

Materials and Methods

All chemicals and tissue culture plates were obtained from the Sigma Chemical Company (St. Louis, MO) and Nalge Nunc International (Naperville, IL), respectively, unless otherwise stated.

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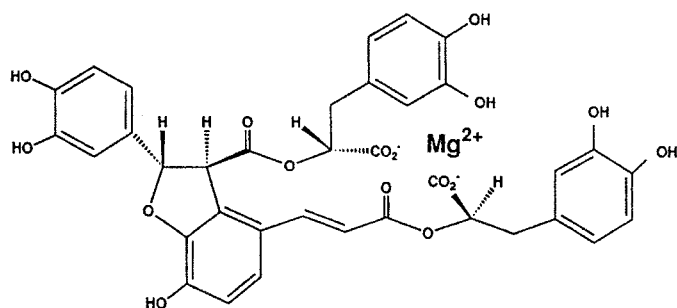


Figure 1. Structural formula of lithospermate B (LAB).

Isolation and Purification of Lithospermate B (LAB)

Magnesium LAB was isolated from *S. miltiorrhizae* as described previously (31).

Induction of Experimental Diabetes Mellitus

Male, 10-wk-old, Sprague-Dawley rats were obtained from the animal facility of Yonsei University College of Medicine, Seoul, Korea. The animals were housed 2 to 3 per cage in an animal room controlled at $23 \pm 2^\circ\text{C}$ and $55 \pm 5\%$ room humidity, under a 12 h-light 12 h-dark cycle. All rats were maintained on standard rat chow (Samyang rat chow, Seoul, Korea) and tap water *ad libitum*.

Diabetes was induced by injecting 60 mg/kg of streptozotocin (STZ) intravenously (dissolved in pH 4.5 citrate buffer immediately before injection) into the tail vein, as described previously (21). Control rats (CR: 10 rats) received the same volume of citrate buffer (2.5 ml/kg). Induction of the diabetic state was confirmed by determining plasma glucose with commercial enzymatic test strips (SureStep, LIFESCAN INC.) two days after the streptozotocin injection. A total of 16 rats with plasma glucose concentrations > 300 mg/dl were defined as diabetic rats. Separate groups of streptozotocin-induced diabetic rats (STZR) and CR were treated with LAB starting 8 wk after the streptozotocin or citrate buffer treatment. LAB was orally administered daily at a dose of 10 mg/kg for an additional 8 wk. The effects of different concentrations of LAB (1 to 20 mg/kg/d) on albuminuria in diabetic STZR were compared during our preliminary study. LAB at dose 10 mg/kg per d exhibited better effect than 1 mg/kg per d but similar effect as 20 mg/kg per d, 10 mg/kg per d was used in further studies. All STZR were survived without insulin treatment in the present study. We did not administer insulin to avoid non-metabolic effects of insulin.

Cell cultures

Murine mesangial cells obtained from the American Type Cell Collection (ATCC: MES-13, cloned from mice transgenic for the early region of the SV-40 virus, passage 25) were grown in DMEM containing 5% fetal bovine serum (FBS), 1% streptomycin-penicillin mixture, 0.5 $\mu\text{g/ml}$ fungizone, 44 mM NaHCO_3 , and 14 mM HEPES in an atmosphere of 5% CO_2 and 95% air at 37°C in a humidified incubator. Results from MES-13 were confirmed by primary rat mesangial cell isolated and cultured as described previously (32). Subcultures were prepared from confluent cultures by trypsinization in Hanks' balanced salt solution containing 0.5 mM EDTA and 0.25% trypsin. These mesangial cells were then grown in DMEM containing 5% FBS to near confluence (80%), and further incubated in serum-free medium for 24 h to arrest and synchronize cell growth. The medium was then changed to serum-free DMEM containing different concentrations of glucose or H_2O_2 in the presence or absence of LAB.

Treatment with LAB up to 40 $\mu\text{g/ml}$ did not show any discernable cytotoxicity, as estimated by the MTT (3-[4,5-Dimethylthiazol-2-yl]-2,5-diphenyl-tetrazolium bromide) assay (control: $100 \pm 7.6\%$; 40 $\mu\text{g/ml}$ LAB treatment: $92.3 \pm 9.1\%$) and LDH release (control: $9.9 \pm 2.4\%$; 40 $\mu\text{g/ml}$ LAB treatment: $12.5 \pm 4.9\%$). Cells were stimulated for 1 h to determine ROS generation or for 48 h for PKC activity and TGF- β 1 and fibronectin protein secretion. We previously demonstrated that the secretion of TGF- β 1 and fibronectin protein by mesangial cells is significantly increased 48 h after the addition of 30 mM glucose (32). After incubation, the media were collected, aliquoted, and frozen at -70°C until required for TGF- β 1 and fibronectin protein determinations. Cellular protein concentrations in cell lysates were measured by the Bio-Rad assay using the Bradford method (33).

Measurement of Urinary Albumin

Urine samples collected over a 24-h period for three consecutive days were centrifuged at $3000 \times g$ for 10 min, and urinary albumin in the supernatants was determined by ELISA using a Nephra microalbumin assay kit (Exocell Inc.). All samples were assayed in triplicate and the mean value from a given rat was calculated.

Measurement of Malondialdehyde (MDA) in the Renal Cortex

A modification of the thiobarbituric acid method of Ohkawa *et al.* (34) was used to measure the level of lipid peroxidation, as described previously (35). The renal cortex was excised and homogenized with extraction solution (iNtron, Seoul, Korea). Aliquots of homogenates (80 μl) were mixed with 80 μl of 8% SDS and a reaction mixture consisting of 120 μl of 0.8% 2-thiobarbituric acid and 120 μl of 20% acetic acid. This solution was placed in a water bath and kept at 95°C for 60 min. After stopping the reaction by cooling with tap water, the mixture was centrifuged at $15,000 \times g$ for 5 min to precipitate interfering particulate materials. The amount of MDA formed was determined by spectrofluorometry (SPF-500C, SLM Instruments) at an emission wavelength of 553 nm at an excitation wavelength of 515 nm and calculated using a tetraethoxypropane standard curve.

Glomerular Histology and Morphometry

For each rat, quantitative morphometry analyses of the different glomerular domains were performed as described previously (36). A 3- μm -thick section was obtained from kidney transversely cut through the hilus in each rat. On this section, 30 different superficial glomeruli were randomly sampled for morphometric analysis as follows: The microscopic slide was scanned clockwise along the superficial cortex, and one glomerulus out of three was analyzed. For every investigated glomerulus, the following measurements were obtained with an automated image analysis system: (1) the total glomerular profile area limited; (2) the glomerular tuft area; (3) the mesangial matrix area (36).

Immunohistochemical Staining for TGF- β 1 and Fibronectin in Renal Cortex

TGF- β 1 and fibronectin were immunolocalized as described previously (37). Fifty-micrometer-thick Vibratome sections taken through the entire kidney were mounted in Epon between polyethylene vinyl sheets. Sections from the cortex were excised and glued on blocks of Epon, and 3- μm sections were cut for light microscopy. Vibratome sections were washed with 50 mM NH_4Cl in PBS three times for 15 min; before incubation with the primary antibody, the sections were incubated for 3 h with PBS that contained 1% bovine

Table 1. General characteristics and the effects of LAB in STZ-induced diabetic rats^a

	CR <i>n</i> = 5	LAB+CR <i>n</i> = 5	STZR <i>n</i> = 8	LAB+STZR <i>n</i> = 8
Plasma glucose (mg/dl)—8 wk	99 ± 9	97 ± 10	493 ± 77 ^b	462 ± 85 ^b
Plasma glucose (mg/dl)—16 wk	97 ± 9	101 ± 8	519 ± 82 ^b	521 ± 97 ^b
Urine volume (ml/24 h)	20 ± 4	20 ± 1	202 ± 79 ^b	203 ± 41 ^b
Kidney weight (g)	2.25 ± 0.13	2.30 ± 0.20	2.35 ± 0.90	2.04 ± 0.23 ^c
Kidney weight/100 g body weight	0.39 ± 0.02	0.39 ± 0.03	0.76 ± 0.14 ^b	0.69 ± 0.08 ^{bc}
Glomerular volume (10 ⁶ μ ³)	1.50 ± 0.36	1.46 ± 0.31	1.72 ± 0.40 ^b	1.44 ± 0.29 ^c

^a Values are means ± SD. CR, citrate buffer injected age-matched control rats; LAB+CR, control rats treated with LAB 10 mg/kg per day; STZR, streptozotocin (60 mg/kg intravenously)—induced diabetic rats; LAB+STZR, streptozotocin-induced diabetic rats treated with LAB 10 mg/kg per day.

^b *P* < 0.05 compared with CR.

^c *P* < 0.05 compared with STZR.

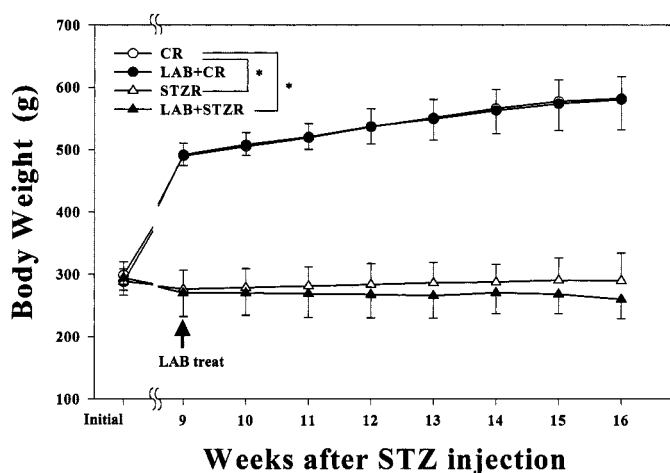


Figure 2. Effects of LAB on body weight of streptozotocin (STZ)-induced diabetic rats. Values are means ± SD. CR; control rats, LAB+CR; control rats treated with LAB 10 mg/kg per d, STZR; streptozotocin-induced diabetic rats, LAB+STZR; streptozotocin induced diabetic rats treated with LAB 10 mg/kg per d. * *P* < 0.05 compared with CR.

serum albumin, 0.05% saponin, and 0.2% gelatin (solution A). The tissue sections then were incubated overnight at 4°C with polyclonal antibody against TGF-β1 and fibronectin obtained from Santa Cruz (Delaware, CA). Antibodies were diluted 1:1000 for TGF-β1 and fibronectin in PBS containing 1% bovine serum albumin (solution B). After several washes in solution A, the sections were incubated for 2 h in peroxidase-conjugated goat anti-rabbit and anti-mouse IgG Fab fragment (Jackson ImmunoResearch Laboratories) diluted 1:100 in solution B. The sections were then rinsed in solution A and subsequently in 0.05 M Trisbuffer (pH 7.6). To detect horseradish peroxidase, the sections were incubated in 0.1% 3,3'-diaminobenzidine in 0.05 M Tris buffer for 5 min. H₂O₂ was then added to a final concentration of 0.01%, and the incubation continued for 10 min. After washing with 0.05 M Tris buffer three times, the sections were dehydrated in a graded series of ethanol, embedded in Epon, and photographed under an Olympus photomicroscope (Tokyo, Japan) that was equipped with differential interference contrast optics.

Analysis of the percentage of area occupied by TGF-β1, and fibronectin positive staining was performed using computer-assisted

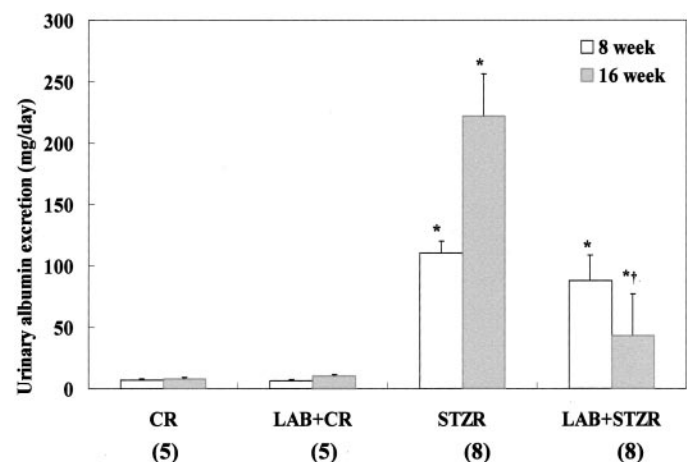


Figure 3. Effect of LAB on urinary albumin excretion in streptozotocin-induced diabetic rats. Numbers in parentheses are number of rats. Urinary albumin was measured by ELISA, as described in Materials and Methods. * *P* < 0.05 compared with corresponding normal control rats at 16 wk, † *P* < 0.05 compared with corresponding diabetic rats at either 8 or 16 wk. CR; control rats, LAB+CR; control rats treated with LAB 10 mg/kg per d, STZR; streptozotocin-induced diabetic rats, LAB+STZR; streptozotocin induced diabetic rats treated with LAB 10 mg/kg per d. Values are means ± SD.

image analysis software (Meta Morph, version 4.6, Universal Imaging Corporation). For each kidney, more than 30 glomerular profiles, cut in equatorial section planes and successively appearing in the visual field of the microscope, were examined.

Staining for Collagen Protein in Renal Cortex: Masson Trichrome Stain

Paraffin-embedded sections were stained with Masson Trichrome for collagen. Extent of collagen accumulation was analyzed using Meta Morph software as described in above.

Assay for TGF-β1 Protein: ELISA

The TGF-β1 in the renal cortex homogenate and in culture media supernatant were converted to the active form by HCl treatment (final concentration, 0.2 M) for 30 min at room temperature followed by neutralization with equimolar NaOH. The amount of TGF-β1 was

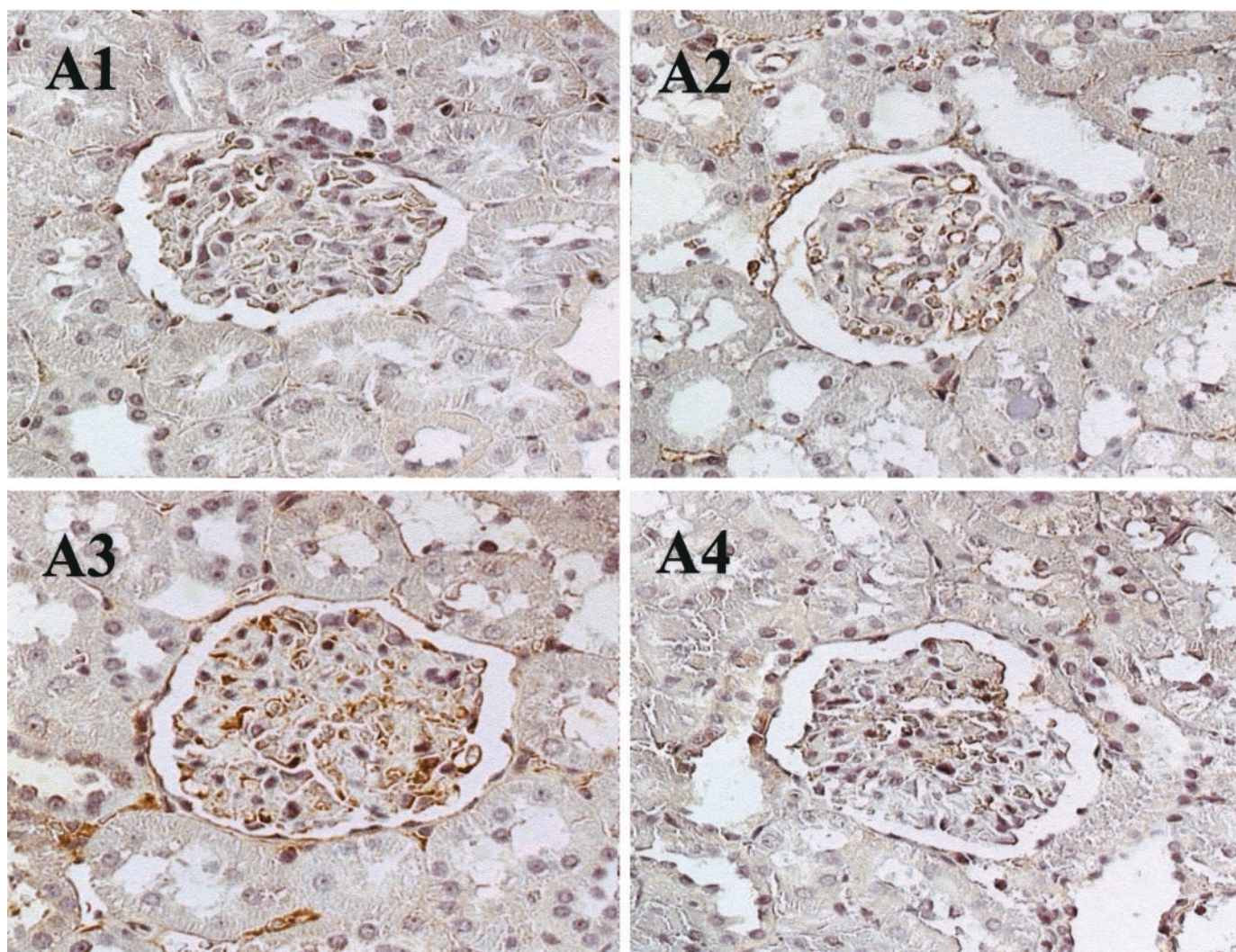


Figure 4. Effects of LAB on renal TGF- β 1 (A), fibronectin (B), and collagen (C) protein expression in streptozotocin-induced diabetic rats. Immunohistochemical staining for TGF- β 1 (A) and fibronectin (B) and Masson trichrome staining for collagen (C) were performed after 8 wk of LAB treatment, as described in Materials and Methods. 1, control; 2, control rats treated with LAB 10 mg/kg per d; 3, streptozotocine-induced diabetic rats; 4, streptozotocine-induced diabetic rats treated with LAB at 10 mg/kg per day. Magnification, $\times 400$.

determined by quantitative sandwich enzyme immunoassay using the TGF- β 1 E_{max} ImmunoAssay System (Promega) according to the manufacturer's description. This TGF- β 1 E_{max} ImmunoAssay System has a detection limit of 32 pg/ml for TGF- β 1.

Assay for Fibronectin Protein: Western Blot Analysis

Immunoblot analysis was used to determine fibronectin in the renal cortex homogenate and in the culture media supernatant as described previously (32). In brief, aliquots of the renal cortex homogenate and conditioned media were mixed with sample buffer containing SDS and β -mercaptoethanol and heated at 95°C for 15 min. Respective samples were then applied to a 5% polyacrylamide gel and subjected to electrophoresis. A prestained SDS-PAGE standard (broad range, Bio-Rad) was used as a molecular weight marker. The proteins were transferred onto a nitrocellulose membrane using a transblot chamber with Tris buffer, and the membranes incubated with rabbit anti-human fibronectin (HRP-conjugated, DAKO, Glostrup, Denmark) diluted 1:10000 in PBS containing 1% bovine serum albumin for 2 h at room temperature and subjected to ECL western blotting (Amersham Life

Science, Little Chalfont, UK). Positive immunoreactive bands were quantified densitometrically and compared with the controls.

Assay for Intracellular ROS

Intracellular ROS production was measured using the method of Bass *et al.* (38) modified for confocal microscopy as described previously (12). In brief, coverglasses of confluent cells obtained 1 h after high glucose stimulation were washed with Dulbecco's PBS and incubated in the dark for 5 min in Krebs-Ringer solution containing 5 mM of 5-(and-6)-chloromethyl-2',7'-dichlorodihydrofluorescein diacetate (CM-H₂DCFDA, Molecular Probes Inc). CM-H₂DCFDA is a nonpolar compound that readily diffuses into cells, where it is hydrolyzed to the nonfluorescent polar derivative 2',7'-dichlorofluorescein (DCF). Culture dishes were transferred to a Leica DM IRB/E inverted microscope, equipped with a $\times 20$ Fluotar objective and a Leica TCS NT confocal attachment, and ROS generation was detected (excitation, 488 nm; emission, 515 to 540 nm). The effect of DCFH phototoxication was minimized by collecting the fluorescence image with a single rapid scan (line average, 4; total scan time, 5.2 s), and identical

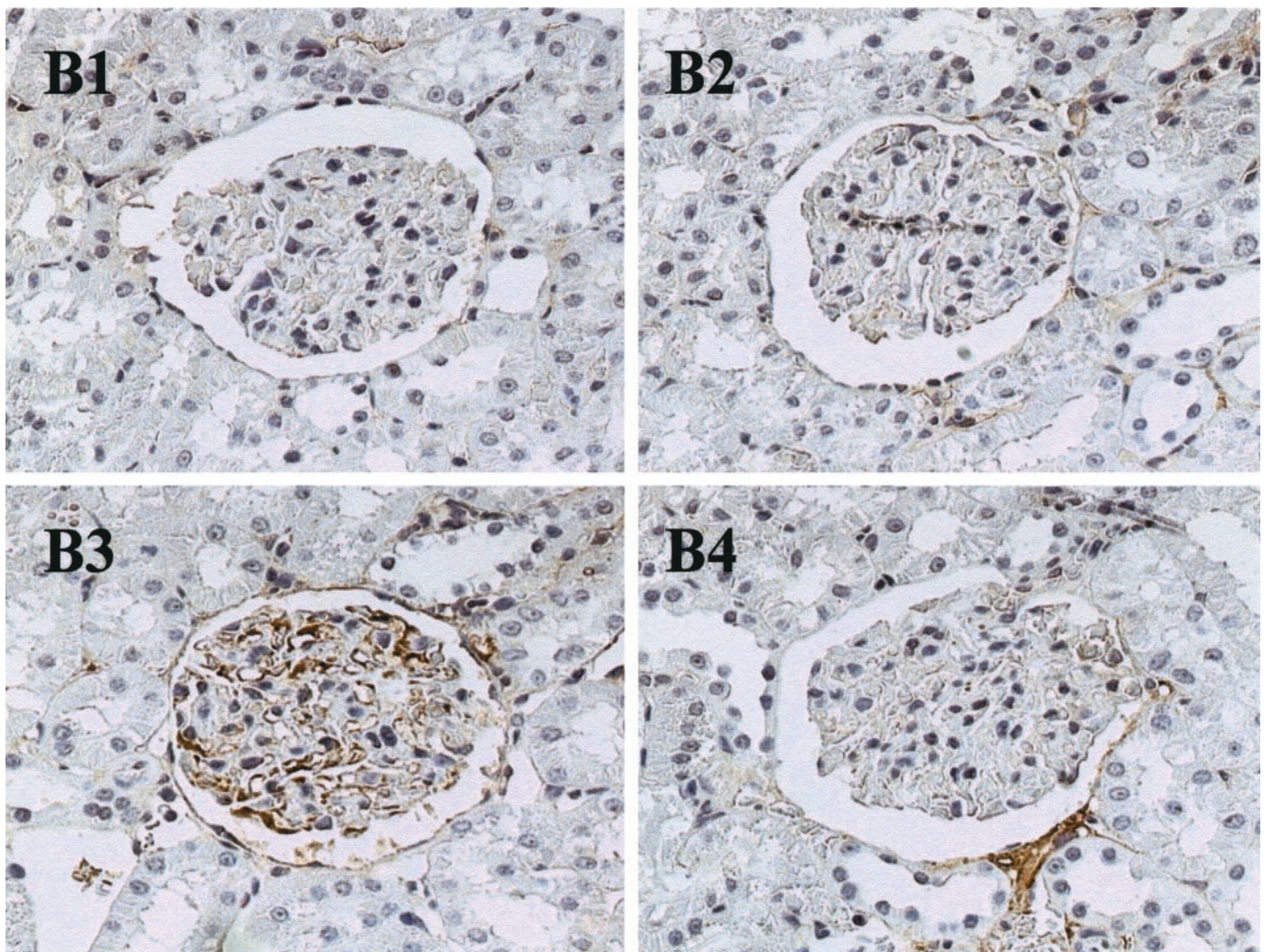


Figure 4. Continued.

parameters, such as contrast and brightness, were used for all determinations. Cells were imaged by differential interface contrast microscopy.

Assay for PKC activity

Membrane and cytosolic fractions were obtained by the method described by Kikkawa *et al.* (13). PKC activities were measured using a Pep Tag Non-Radioactive Assay kit (Promega). Fluorescence peptide (D-L-S-R-T-L-S-V-A-A-K), which is highly specific for PKC, was used as a substrate. The phosphorylation of this specific substrate by PKC alters the peptide's net charge from +1 to -1. The phosphorylated substrate so obtained was separated in agarose gel at pH 8.0, and negatively charged bands from the gel were removed and heated at 95°C until the gel slice melted. Absorbance of the solubilized slice was measured using a spectrophotometer at 570 nm.

To investigate whether LAB acts as a direct inhibitor of PKC, LAB (5 to 200 µg/ml) was added directly to reaction mixtures containing rat brain PKC supplied by manufacturer or membrane fractions of mouse mesangial cell cultured under high glucose for 48 h.

Statistical Analysis

All results are expressed as means \pm SD. ANOVA was used to assess the intergroup differences. If the F statistics proved to be

significant, the mean values obtained from each group were compared using Fisher least significant difference method. A P value of less than 0.05 was used as the criterion for statistically significant difference.

Results

Effects of LAB on the Renal Function of STZR

Rats with plasma glucose concentrations of more than 300 mg/dl at 2 d after STZ injection were used for the present study. As summarized in Table 1 and Figure 2, STZR failed to gain body weight compared with the control rats (CR) and exhibited polyuria. The absolute mean kidney weight of STZR was similar to the CR. However, mean kidney weight of the STZR expressed as a function of body weight was significantly higher than that of CR. STZR also exhibits glomerular hypertrophy. Urinary albumin excretion was found to be significantly increased 8 wk after STZ injection and continuously increased up to 16 wk after STZ injection (Figure 3).

The administration of LAB at 10 mg/kg per d did not affect plasma glucose levels in either the control or the diabetic rats. Although the mean body weight of LAB+STZR was slightly lower than that of STZR, the difference was not statistically

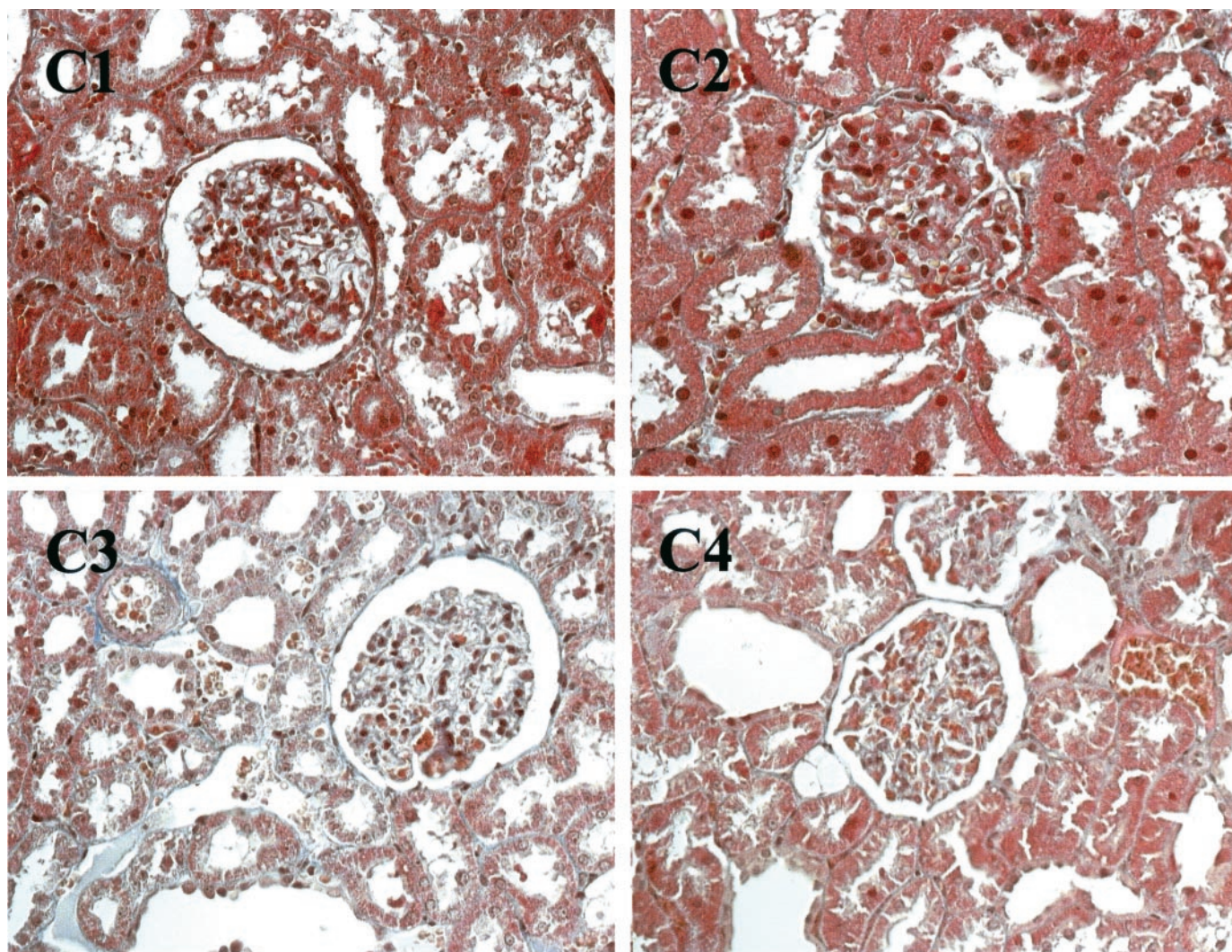


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significant (Figure 2). The plasma glucose of LAB+STZR was similar as that of STZR, it is unlikely that LAB decreased food consumption in diabetic rats. LAB used in the present study did not affect SGOT, SGPT, and survival of diabetic as well as control rats.

The administration of LAB, started 8 wk after STZ treatment for 8 wk significantly ameliorated absolute kidney weight, kidney weight expressed as a function of body weight, glomerular hypertrophy (Table 1), and albuminuria (Figure 3) in STZR, although kidney weight expressed as a function of body weight and urinary albumin excretion in LAB+STZR remained statistically higher than in CR.

Immunostaining for TGF- β 1 (Figure 4A) and fibronectin (Figure 4B) and Masson Trichrome staining for collagen (Figure 4C) demonstrated that increases in TGF- β 1, fibronectin, and collagen deposition in both glomeruli and tubulointerstitium of the renal cortex in diabetic STZR. Semiquantitative analyses for each parameter in the glomeruli and in the tubules from different experimental group are summarized in Table 2. Delayed treatment with LAB effectively inhibited all diabetes-

associated TGF- β 1, fibronectin, and collagen upregulation in the renal cortex. Upregulation of TGF- β 1 and fibronectin in the cortex were confirmed by ELISA and Western blot analysis, respectively (Figure 5, A and B).

Quantitative morphometry performed by automated image analysis showed a diabetes-related increase in a mesangial expansion (Table 2). The mesangial area increased by 2.3-fold, and LAB treatment reversed mesangial expansion (Table 2).

As summarized in Figure 6, MDA was sevenfold higher in the renal cortex of STZR than in CR at 16 wk after STZ treatment. MDA in LAB+STZR was significantly lower than in STZR but statistically higher than in CR and LAB+CR.

Effects of LAB on TGF- β 1 and Fibronectin Protein Secretion by Mesangial Cells Cultured under High Glucose or H_2O_2

Quantitative analyses of TGF- β 1 showed that the exposure of mesangial cells to 30 mM glucose (Figure 7A) or 100 μ M H_2O_2 (Figure 7B) significantly induced TGF- β 1 protein secretion after 48 h. LAB inhibited high glucose-induced TGF- β 1

Table 2. Morphometric studies for kidney cortex (% of positive area)^a

	CR <i>n</i> = 5	LAB+CR <i>n</i> = 5	STZR <i>n</i> = 5	LAB+STZR <i>n</i> = 5
Glomerular TGF- β 1	1.69 \pm 0.75	1.34 \pm 1.12	10.33 \pm 4.36 ^b	2.73 \pm 1.33 ^{bc}
Tubulointerstitial TGF- β 1	0.92 \pm 0.41	0.82 \pm 0.49	3.15 \pm 1.25 ^b	0.93 \pm 0.74 ^c
Glomerular fibronectin	1.43 \pm 0.92	1.35 \pm 0.92	9.99 \pm 5.15 ^b	2.37 \pm 1.42 ^c
Tubulointerstitial fibronectin	0.72 \pm 0.46	0.68 \pm 0.61	3.02 \pm 1.46 ^b	0.98 \pm 0.81 ^c
Glomerular collagen	3.09 \pm 1.84	3.19 \pm 1.45	11.67 \pm 3.62 ^b	3.16 \pm 1.50 ^c
Tubulointerstitial collagen	1.32 \pm 0.52	1.54 \pm 0.52	6.25 \pm 2.20 ^b	1.98 \pm 0.72 ^{bc}
Mesangial matrix fraction (%)	8.1 \pm 3.4	9.6 \pm 3.5	18.8 \pm 4.7 ^b	14.1 \pm 5.2 ^c

^a Values are means \pm SD. CR, citrate buffer injected age-matched control rats; LAB+CR, control rats treated with LAB 10 mg/kg per day; STZR, streptozotocin (60 mg/kg intravenously)—induced diabetic rats; LAB+STZR, streptozotocin-induced diabetic rats treated with LAB 10 mg/kg per day. Semiquantitative analysis for area stained for TGF- β 1, fibronectin, and collagen were performed as described in the method.

^b $P < 0.05$ compared with CR.

^c $P < 0.05$ compared to STZR.

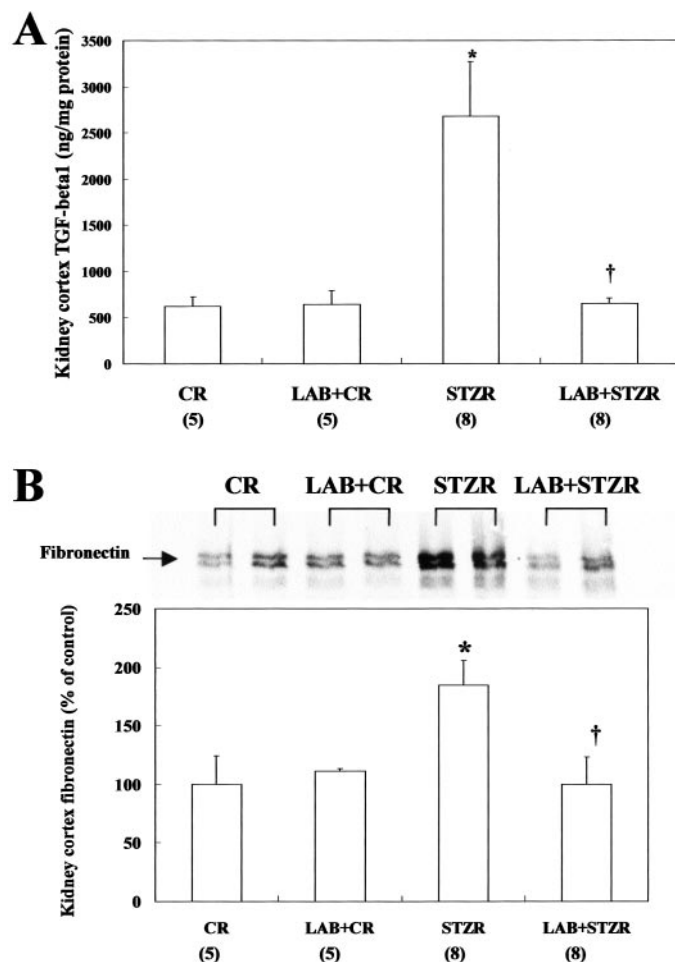


Figure 5. Effects of LAB on renal TGF- β 1 and fibronectin protein expression in streptozotocin-induced diabetic rats. ELISA for TGF- β 1 (A) and Western blot for fibronectin (B) were performed after 8 wk of LAB treatment, as described in Materials and Methods. Numbers in parentheses are number of rats. * $P < 0.05$ compared with normal control rat; † $P < 0.05$ compared with diabetic rats. CR, control rats; LAB+CR, control rats treated with LAB 10 mg/kg per d; STZR, streptozotocin-induced diabetic rats; LAB+STZR, streptozotocin induced diabetic rats treated with LAB 10 mg/kg per d. Values are means \pm SD.

secretion by mesangial cells in a dose-dependent manner (Figure 7A). LAB at above 20 μ g/ml decreased TGF- β 1 protein secretion by mesangial cells cultured in control 5.6 mM glucose (data not shown), 10 μ g/ml of LAB was used to determine the effects on H₂O₂-induced TGF- β 1 secretion (Figure 7B).

The effects of LAB on high glucose- or H₂O₂-induced fibronectin secretion were qualitatively similar to the changes observed in TGF- β 1 secretion. Exposure of mesangial cells to 30 mM glucose (Figure 8A) or 100 μ M H₂O₂ (Figure 8B) significantly increased fibronectin secretion after 48 h. LAB at 10 μ g/ml inhibited both high glucose- and H₂O₂-induced, but not basal, fibronectin secretions by mesangial cells.

LAB also inhibited high glucose-induced fibronectin up-regulation in primary rat mesangial cells. Fibronectin secretion under control, 30 mM glucose, and 30 mM glucose in the presence of 10 μ g/ml LAB were 100, 161 \pm 15 ($P < 0.05$

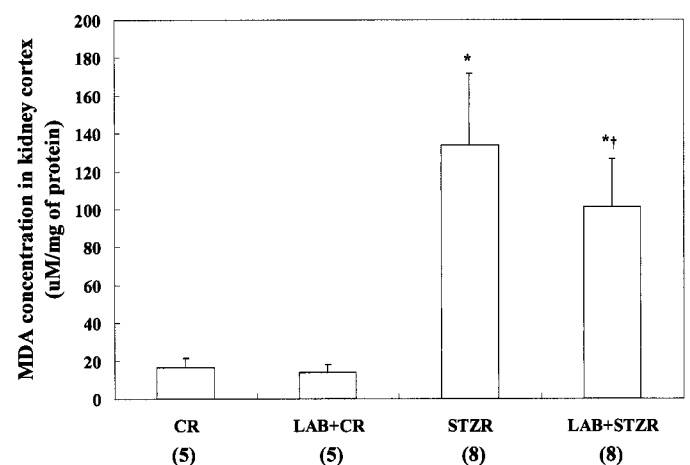


Figure 6. Effect of LAB on malondialdehyde (MDA) in the renal cortex of streptozotocin-induced diabetic rats. Values are means \pm SD. * $P < 0.05$ compared with normal control; † $P < 0.05$ compared with diabetic rats. CR, control rats; LAB+CR, control rats treated with LAB 10 mg/kg per d; STZR, streptozotocin-induced diabetic rats; LAB+STZR, streptozotocin-induced diabetic rats treated with LAB at 10 mg/kg per d.

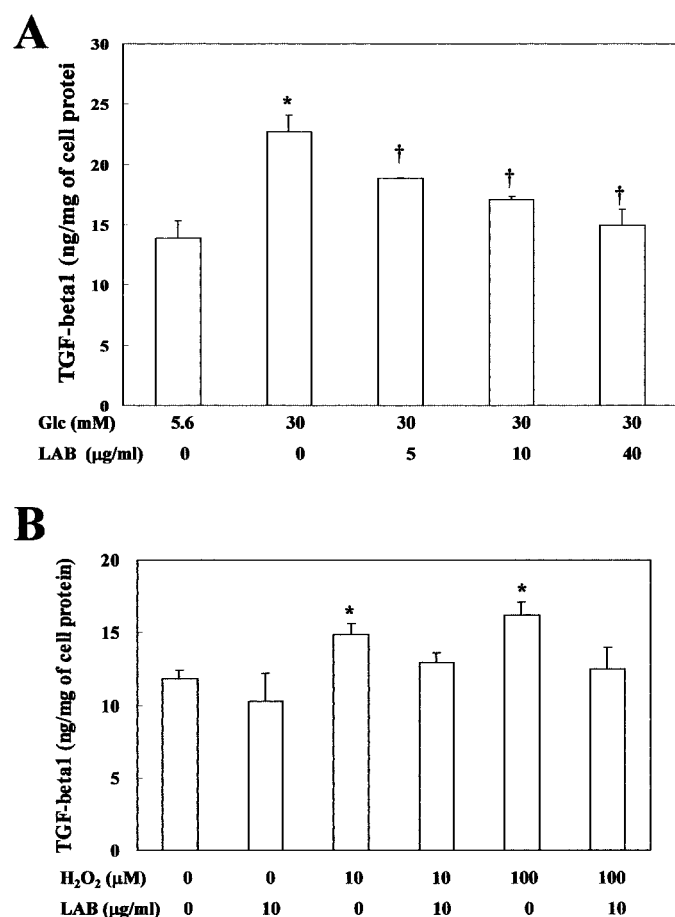


Figure 7. ELISA for TGF- β 1 protein secreted by mesangial cells. Growth arrested and synchronized mesangial cells were grown in different concentrations of LAB containing either 5.6 mM of D-glucose + 24.4 mM mannitol or 30 mM of D-glucose (A) or in different concentrations of H₂O₂ in the presence or absence of LAB (B) for 48 hours. Values represent means \pm SD obtained from four individual experiments; * $P < 0.05$ compared with 5.6 mM D-glucose + 24.4 mM mannitol; † $P < 0.05$ compared with 30 mM D-glucose.

versus control), and $74 \pm 11\%$ ($P < 0.05$ versus 30 mM glucose), respectively.

Effects of LAB Intracellular ROS in Mesangial Cells Cultured under High Glucose or H₂O₂

The incubation of mesangial cells with 30 mM of glucose for 1 h (Figure 9A) or H₂O₂ for 15 min (Figure 9B) increased intracellular ROS. Quantitative analyses of ROS showed that the exposure of mesangial cells to high glucose increased intracellular ROS 11-fold compared with control and that this was effectively inhibited by the addition of LAB 10 μ g/ml; 100 μ M of H₂O₂ increased intracellular ROS tenfold compared with control, and this was also effectively inhibited by the addition of LAB at 10 μ g/ml.

Effects of LAB on PKC Activity in Mesangial Cells Cultured under High Glucose

As shown in Figure 10, 30 mM of glucose did not affect PKC activity in the cytosolic fraction of mesangial cells but

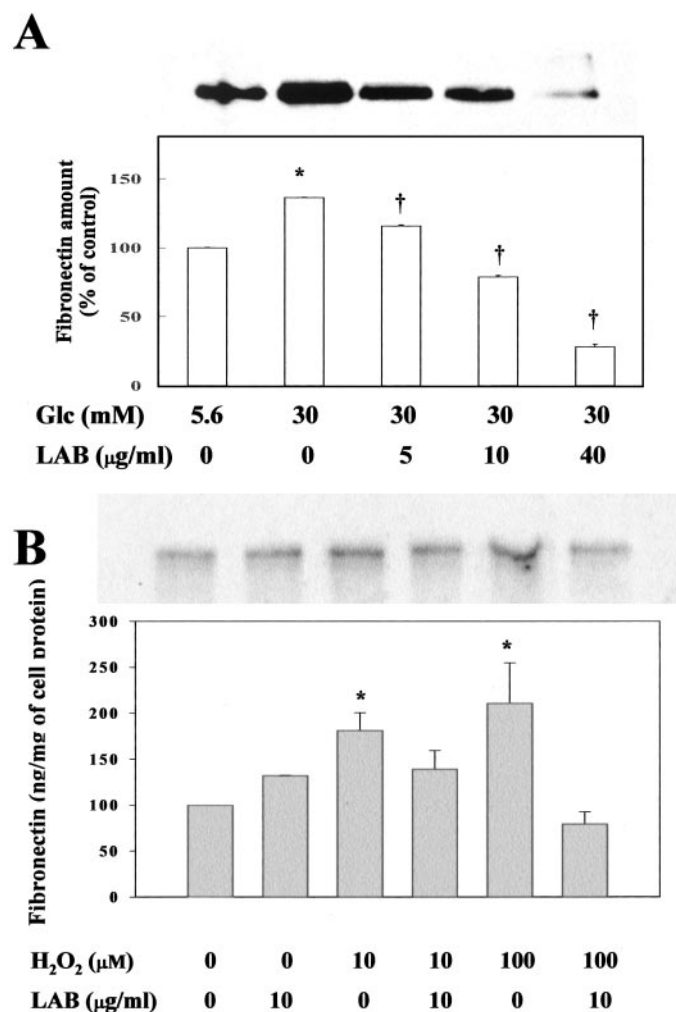


Figure 8. Western blots of fibronectin protein secreted by mesangial cells. Growth arrested and synchronized mesangial cells were grown in different concentrations of LAB containing either 5.6 mM D-glucose + 24.4 mM mannitol or 30 mM of D-glucose (A) or in different concentrations of H₂O₂ in the presence or absence of LAB (B). Values represent means \pm SD obtained from four individual experiments. * $P < 0.05$ compared with 5.6 mM D-glucose + 24.4 mM mannitol control; † $P < 0.05$ compared with 30 mM glucose.

increased PKC activity in the membrane fraction, suggesting that high glucose induced PKC activation under our experimental conditions. Increased PKC activity in the membrane fraction of mesangial cells cultured under 30 mM glucose condition was inhibited by LAB in a dose-dependent manner (Figure 10).

LAB also inhibited high glucose-induced membrane PKC activity upregulation in primary rat mesangial cell. The PKC activity in control, 30 mM glucose, and 30 mM glucose + 10 μ g/ml LAB were 100, 251 ± 14 ($P < 0.05$ versus control), and $176 \pm 27\%$ ($P < 0.05$ versus 30 mM glucose), respectively.

As summarized in Figures 11A and 11B, exogenously administered LAB into purified rat brain PKC or membrane fraction of mouse mesangial cell did not inhibit PKC activity at concentrations up to 40 μ g/ml. Concentrations of LAB higher

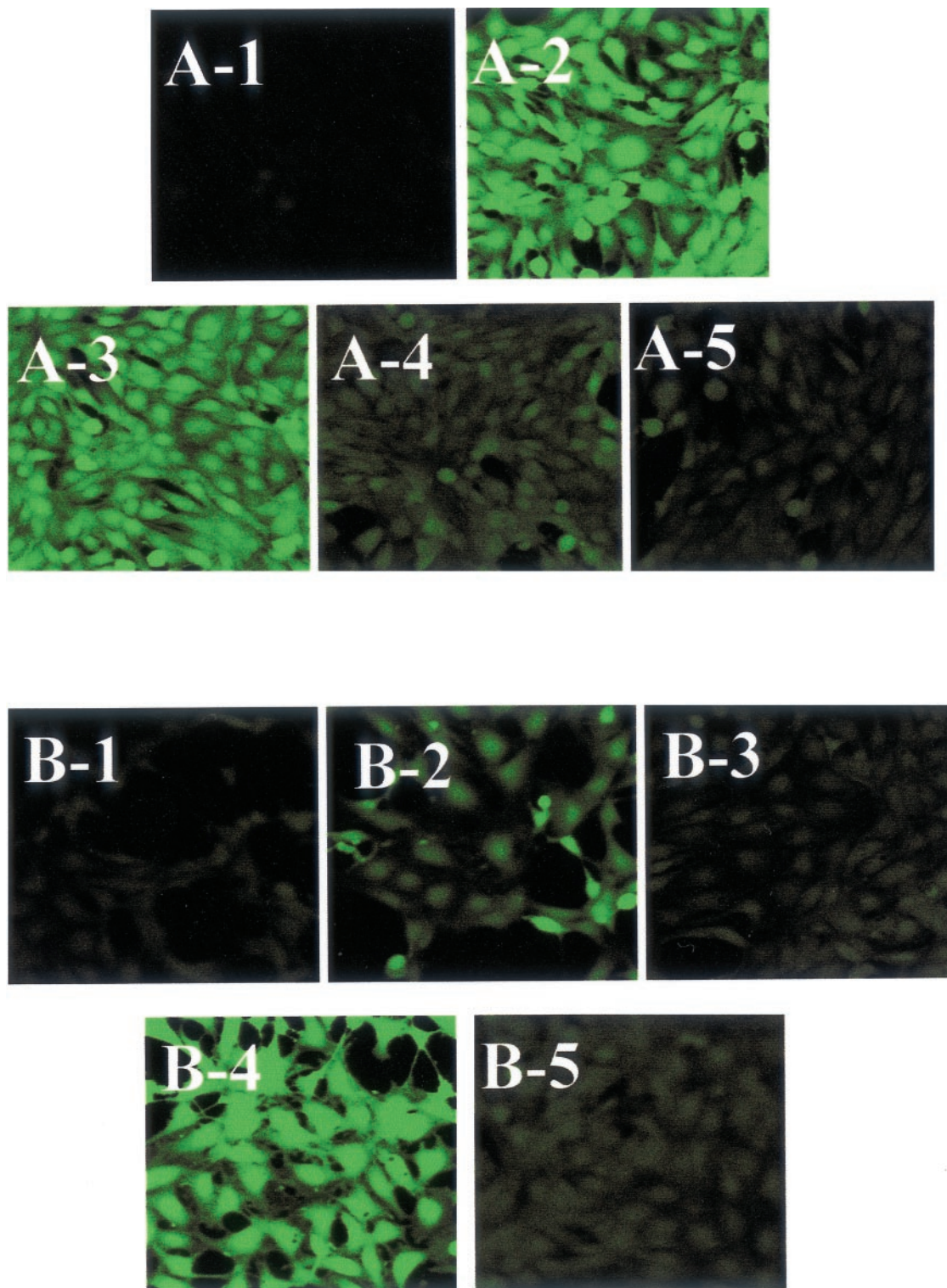


Figure 9. Effect of LAB on high glucose- (A) and H_2O_2 - (B) induced dichlorofluorescein-sensitive intracellular reactive oxygen species in mesangial cells. Growth arrested and synchronized quiescent mesangial cells, grown on coverglasses, were incubated in 5.6 mM D-glucose + 24.4 mM mannitol or 30 mM of D-glucose in the presence or in the absence of LAB for 1 h, washed with PBS, and incubated in the dark for 10 min in Krebs-Ringer solution containing 5 mM of 5-(and-6)-chloromethyl-2',7'-dichlorodihydrofluorescein diacetate (CM- H_2DCFDA). Culture dishes were transferred to a Leica DM IRB/E inverted microscope, equipped with a $\times 20$ Fluotar objective and a Leica TCS NT confocal attachment, and ROS generation was visualized. A-1: 5.6 mM D-glucose + 24.4 mM mannitol control; A-2: 30 mM glucose; A-3: 30 mM glucose + 5 $\mu\text{g/ml}$ LAB; A-4: 30 mM glucose + 10 $\mu\text{g/ml}$ LAB; A-5: 30 mM glucose + 40 $\mu\text{g/ml}$ LAB; B-1: 5.6 mM D-glucose; B-2: 5.6 mM glucose + 10 μM H_2O_2 ; B-3: 5.6 mM glucose + 10 μM H_2O_2 + 10 $\mu\text{g/ml}$ LAB; B-4: 5.6 mM glucose + 100 μM H_2O_2 ; B-5: 5.6 mM glucose + 100 μM H_2O_2 + 10 $\mu\text{g/ml}$ LAB. Representative data from four individual experiments.

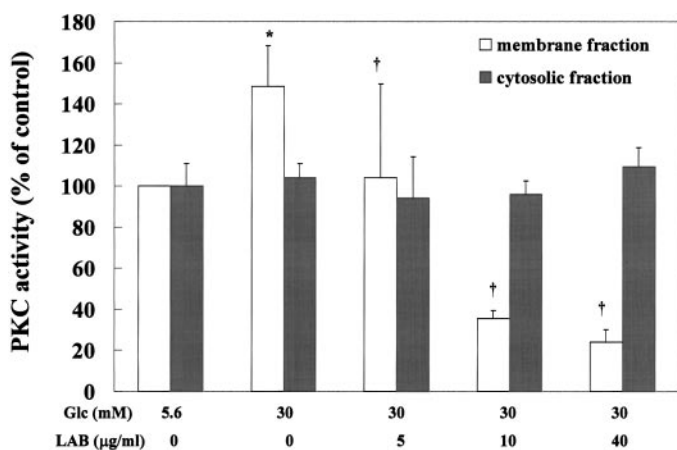


Figure 10. Effect of LAB on high glucose-induced PKC activation in mesangial cells. Growth arrested and synchronized quiescent mesangial cells grown on plates were incubated with 5.6 mM D-glucose + 24.4 mM mannitol or 30 mM of D-glucose in the presence or in the absence of LAB for 24 h, washed with PBS, and harvested. PKC activities were measured using a Pep Tag Non-Radioactive Assay kit for PKC. The fluorescent peptide D-L-S-R-T-L-S-V-A-A-K, which is highly specific for PKC, was used as a substrate. Values are means \pm SD obtained from four individual experiments. * $P < 0.05$ compared with 5.6 mM D-glucose + 24.4 mM mannitol control, † $P < 0.05$ compared with 30 mM glucose control.

than 100 $\mu\text{g/ml}$ directly inhibited PKC activity in a dose-dependent manner.

Discussion

The efficacy of antioxidant supplementation when they were administered at the onset of experimental diabetes has been reported to prevent diabetic renal injury (19,21–26). The present study demonstrates that delayed treatment with LAB at a dose that reduces renal MDA accumulation significantly suppresses the progression of albuminuria, glomerular hypertrophy, mesangial expansion, and upregulation of renal TGF- β 1, fibronectin, and collagen expression in STZR. In view of the fact that in the clinical situation treatment is often initiated after the onset of diabetic nephropathy, the present finding suggests that LAB may prove to be a new therapeutic agent for the treatment of diabetic nephropathy.

Renal TGF- β 1, fibronectin, and collagen upregulations in STZR were totally blocked by delayed LAB treatment. The levels of urinary albumin excretion and MDA in the renal cortices of LAB+STZR were also significantly lower than that of the STZR but statistically higher than those of CR in the present study. These differences may have resulted from the different time courses among TGF- β 1, fibronectin, and collagen upregulation in the renal cortex, albuminuria, or MDA accumulation in STZR and/or the sensitivity of each measurement used in present study. The concentration of MDA and the expression of TGF- β 1, fibronectin, and collagen in renal cortex at 8 wk after STZ injection but before treatment with LAB could not be measured. UAE in diabetic rats in the present study are similar to those in a previous study (39).

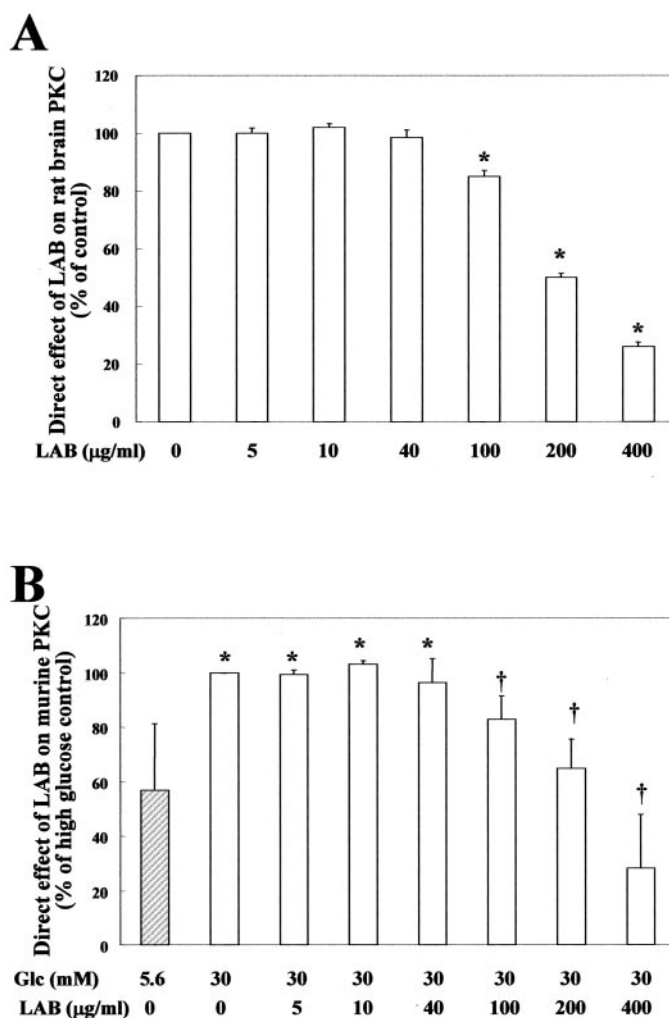


Figure 11. Direct effect of LAB on PKC activity. To investigate whether LAB acts as a direct inhibitor of PKC, LAB (5 to 400 $\mu\text{g/ml}$) was added directly to reaction mixtures containing rat brain PKC (A) or containing membrane fractions of mouse mesangial cell that were incubated under high glucose conditions for 48 h (B). * $P < 0.05$ compared with control; † $P < 0.05$ compared with high glucose. Values are means \pm SD.

We, therefore, examined the antioxidative property of LAB by using of mesangial cells cultured under high glucose, an accepted *in vitro* model for diabetic nephropathy. LAB inhibited high glucose-induced intracellular ROS, and LAB, at a dose-inhibiting ROS generation, inhibited both high glucose- and H_2O_2 -induced TGF- β 1 and fibronectin protein secretion by mesangial cells; therefore, the protective effects of LAB on experimental diabetic nephropathy may be largely due to its antioxidative effects.

The inhibition of H_2O_2 -induced intracellular DCF-sensitive fluorescence by LAB (Figure 9B) is in general agreement with previous reports, which demonstrated that *S. multiarhizae* normalizes catalase activity and restores superoxide dismutase levels (30). Previous studies have demonstrated that high glucose increases intracellular ROS as a result of glucose uptake and metabolism in mesangial cells (12) and found that different

antioxidants ameliorate high glucose-induced TGF- β 1 and ECM synthesis in mesangial cells (18,20). In agreement with these studies, we found that LAB, at concentrations that inhibited high glucose-induced intracellular ROS generation, effectively blocked TGF- β 1 and fibronectin upregulation in mesangial cells cultured under high glucose conditions.

Although LAB treatment inhibited high glucose-induced PKC activation in mesangial cells, LAB at concentrations up to 40 μ g/ml, did not directly inhibit PKC activity. This suggests that the inhibition of high glucose-induced PKC activation by LAB may occur indirectly through the inhibition of intracellular ROS generation. PKC activation under hyperglycemic conditions is largely related to an increase in the *de novo* synthesis of diacylglycerol (DAG), the major endogenous activator of PKC, and results from an increased glucose metabolism (10). However, PKC can be sensitively regulated by redox modification, which is independent of DAG. The N-terminal regulatory domain of PKC contains zinc-binding, cysteine-rich motifs that are susceptible to oxidative modification, and which are readily oxidized by peroxide. When oxidized, the autoinhibitory function of the regulatory domain is compromised and cellular PKC activity is consequently stimulated. In addition, H₂O₂ activates PKC- δ through the activation of tyrosine kinase (40). In this context, a recent study by Nishikawa *et al.* (7) demonstrated that ROS play an important role in PKC activation in endothelial cells cultured under high glucose conditions. PKC activations in mesangial cells cultured under high glucose conditions (20) and in STZ-induced diabetic rats (41) were effectively inhibited by antioxidants.

Although the chemical structure of LAB is known (Figure 1), it is not clear at present whether mesangial cells take up LAB. Further studies are needed to determine the exact mechanisms involved in inhibition of intracellular ROS by LAB.

Potential toxic effects of LAB should also be investigated in detail, because the mean body weight of LAB+STZR was slightly lower, although it is not statistically different, than that of STZR (Figure 2). Data from the present study provided that LAB did not affect survival, SGOT, and SGPT in diabetic STZR as well as CR (data not shown).

In conclusion, LAB inhibited the ROS generation, and this led to PKC activation and TGF- β 1 and fibronectin upregulation in mesangial cells cultured under high glucose conditions. Moreover, delayed treatment with LAB significantly suppressed the progression of renal injury in diabetic rats. LAB, an active component of *S. miltiorrhizae* radix, may prove to be a new therapeutic agent for the treatment of diabetic nephropathy.

Acknowledgments

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